Early changes in bacterial communities in wound tissues of *Pinus massoniana* after inoculation with *Bursaphelenchus xylophilus*

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Summary – The bacterial communities in the wound tissues of *Pinus massoniana* were analysed by 16S rDNA amplicon sequencing. The results showed that the bacterial richness and diversity changed remarkably whether the wound was inoculated with pine wood nematode (PWN; *Bursaphelenchus xylophilus*) or not after 12 h. However, the predominant bacteria *Stenotrophomonas*, Burkholderiaceae, *Pseudomonas*, *Serratia* and *Delftia*, introduced by PWN in the wound tissues, changed within 6 h. After 6 h of PWN inoculation, the most abundant genus associating with PWN, *Stenotrophomonas*, failed to colonise the wound tissues, and the abundance of *Delftia* decreased, while the other representative bacteria, Burkholderiaceae, *Pseudomonas* and *Serratia*, from the PWN were markedly enriched. In addition, our study is the first to report the association of *Serratia liquefaciens* with PWN. Predicted functional analyses using the Tax4Fun tool showed that the alterations in bacterial composition also led to shifts in their functional pathways, especially after 12 h of PWN inoculation. These findings clarified that the bacteria carried by PWN were responsible for the alterations in bacterial communities in the wound tissues and will shed light on the invasion mechanism of PWN.

Keywords – 16S rDNA, bacterial diversity, Burkholderiaceae, *Delftia*, pine wilt disease, pine wood nematode, *Pseudomonas*, *Serratia*, *Serratia liquefaciens*, *Stenotrophomonas*.

Pine wilt disease (PWD) is one of the most destructive diseases of pine trees in the world. The causal agent of the disease was initially confirmed as *Bursaphelenchus xylophilus* (Steiner & Buhner, 1934), the pine wood nematode (PWN), by Kiyohara & Tokushige (1971) and for years it was thought to be the only pathogenic agent (Mamiya, 1975, 1983; Nickle *et al.*, 1981; Fukuda *et al.*, 1992). However, some scientists studying the mechanisms of PWD attributed the rapid wilting of pine trees to wilt toxins, and the toxins were probably produced by nematode-associated bacteria (Oku *et al.*, 1979, 1980; Kawazu *et al.*, 1996; Han *et al.*, 2003; Zhao *et al.*, 2003; Tan & Feng, 2004; Guo *et al.*, 2006; Yin *et al.*, 2007). As a result, many experiments have been conducted on these bacteria, including surface bacteria and endobacteria of PWN (Zhao *et al.*, 2000; Wu *et al.*, 2013; Tian *et al.*, 2015), as well as bacteria associated with the beetle vectors (Vicente *et al.*, 2013a; Alves *et al.*, 2016) and hosts of PWN (Proença *et al.*, 2017a), aiming to clarify

the origin and exact role of bacteria in PWD. As reviewed by Proença *et al.* (2017b), the bacteria *Pseudomonas*, *Burkholderia*, *Serratia*, *Ewingella* and *Enterobacter* were most commonly reported to be associated with PWD in China, Korea, Portugal and the USA, and other genera, such as *Stenotrophomonas*, *Bacillus* and *Pantoea*, were also carried by the PWN. However, despite the intense research and abundant information about the possible functions of these bacteria, the role of associated bacteria in PWD has mostly been tested *in vitro*, and when these bacteria come into play in PWD progression remains unclear.

In the wild, the PWN is transmitted from tree to tree by insect vectors, mostly from the genus *Monochamus* (Coleoptera: Cerambycidae), including *Monochamus alternatus* in East Asia (Mamiya & Enda, 1972; Lee *et al.*, 1990; Ning *et al.*, 2004), *M. carolinensis* in North America (Linit *et al.*, 1983) and *M. galloprovincialis* in Portugal (Naves *et al.*, 2001), through feeding wounds (Linit,

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1990) or oviposit wounds (Edwards & Linit, 1992). Afterwards, the PWN moves from the trachea to the tail tip of the vectors and is finally transmitted to the wounds of pine twigs (Aikawai & Tiogashi, 1998), where a new infection cycle begins. Nevertheless, the nematodes hardly migrate or migrate slowly on the wound surface (Tamura, 1984) because most of them are trapped in the sticky resin exuded by epithelial cells on the wound surface (Zhao, 2008) and have to survive harmful secondary metabolites produced by defence mechanisms of the host (Cheng *et al.*, 2013) before successfully invading pine tissues. In this initial stage, some bacteria in the wound may already begin to react against the defence metabolites of the host and help PWN tolerate and overcome the resistance of the host. Therefore, determining what species of bacteria are the pioneers that play a role in this stage is important for illuminating the invasion mechanism of PWN.

To answer the questions mentioned above, the aim of this study was to analyse the changes in bacterial communities in wound tissues caused by inoculation of PWN in the initial stage using 16S rDNA amplicon sequencing, aiming to clarify the bacterial pioneers that would affect the successful invasion of PWN.

Materials and methods

EXPERIMENTAL MATERIALS

A virulent PWN strain was initially isolated from naturally infected *Pinus massoniana* (Fujian, P.R. China). After morphological identification, the nematodes were propagated on *Pestalotiopsis* sp. (Xie *et al.*, 2017a, b; Sriwati *et al.*, 2008) cultured on PDA medium at 28°C for 7 days. The harvested nematodes were then collected using a Baermann funnel. Large quantities of nematodes were obtained based on repetitive propagation using this method and stored at 4°C until use. Three-year-old *P. massoniana* seedlings with similar growing conditions (height, 70–80 cm; diam., 1 cm), planted in the Institute of Forest Protection in Fujian Agriculture and Forestry University, China, were used in the inoculation experiment.

INOCULATION AND SAMPLING

The cultured nematodes were rinsed three times with sterile deionised water before use and adjusted to 30 μ l nematode suspension (approximately 1000 nematodes) in each 0.5 ml PCR tube; this solution was then directly pipetted onto the artificial wounds of the seedlings. Each

seedling had five artificial wounds that were generated by scraping the bark with a razor blade. The longitudinal and axial lengths of the wounds were 3 cm and 0.5 cm, respectively, and the wounds were 5 cm apart from each other. The plants inoculated with the same amount of sterile water were used as controls. To distinguish the origin of bacteria in the wound tissues after inoculation, the bacterial communities carried by the PWN and the communities that originally existed in the cortex tissues of healthy *P. massoniana* seedlings were detected.

As most of the nematodes were trapped on the wound surface for approximately 6–12 h according to different host and inoculum densities (Jin, 2007; Zhang *et al.*, 2007; Li & Ye, 2008; Su *et al.*, 2008), we chose 6 h and 12 h as the experimental time points. The tissues of the wounds, mostly the cortex, were collected 6 and 12 h after inoculation and then stored at –80°C until use, as were the corresponding two control groups. The cortex tissues of healthy *P. massoniana* seedlings were collected immediately after the bark had been scraped and the rest of the cultured nematodes (three tubes with 0.5 ml nematode precipitate in each) were rinsed three times with sterile deionised water before storing at –80°C. Because of the limited amount of cortex tissues from one single wound, a mixture of 15 cortex tissues from 15 wounds (three seedlings) was treated as a treatment sample, and each treatment had three replicates (nine seedlings). Thus, a total of 45 pine seedlings were used in this research, with nine seedlings in each group (two experimental groups, two control groups and one group of healthy pine tissues). The tools used above were all decontaminated before use, and the experiment was conducted at 28°C in a glasshouse.

DNA EXTRACTION

Total genomic DNA of the samples was extracted using the CTAB method (Lutz *et al.*, 2011), and the concentration and purity were detected by 1% agarose gel electrophoresis. Afterwards, the DNA products were diluted to 1 ng μ l^{–1} with sterile water before PCR amplification.

PCR AMPLIFICATION

To study the diversity and composition of bacteria in the wound tissues, the distinct V3–V4 regions of 16S rDNA were PCR amplified with specific barcoded primers: 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGTATCTAAT-3'). PCR was performed

in a 30 μ l volume, containing 15 μ l Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.1 μ M each primer and 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min.

GENE LIBRARY PREPARATION AND SEQUENCING

PCR products were detected using 2% agarose gel electrophoresis, and then purified with a GeneJET Gel Extraction Kit (Thermo Scientific). Sequencing libraries were generated using the Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific) following the manufacturer's recommendations. After assessing the library quality on the Qubit® 2.0 Fluorometer (Thermo Scientific), the PCR products were sequenced on an Ion S5™ XL platform, and 600 bp single-end reads were generated.

BIOCHEMICAL ANALYSIS

Raw reads were obtained after single-end reads were assigned to samples based on their unique barcode and truncated by removing the barcode and primer sequences. Quality filtering of the raw reads was performed under specific filtering conditions to obtain high-quality clean reads according to the Cutadapt (Version 1.9.1, <http://cutadapt.readthedocs.io/en/stable/>) (Martin, 2011) quality control process. Subsequently, clean reads were finally obtained after comparison with the Silva database (<https://www.arb-silva.de/>) (Quast *et al.*, 2012) using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) (Edgar *et al.*, 2011) to detect and remove the chimeric sequences.

Sequence analysis was performed by UPARSE software (Version 7.0.1001, <http://drive5.com/uparse/>) (Edgar, 2013). Sequences with $\geq 97\%$ similarity were clustered to the same operational taxonomic units (OTUs). A representative sequence for each OTU was screened for further annotation, and taxonomic information was annotated by means of the Silva database based on the MOTHUR algorithm.

To assess microbial diversity with or without infection by PWN, alpha diversity was calculated, including observed species, Chao 1, Shannon, Simpson and good coverage, all of which were calculated with QIIME (Version 1.7.0) (Caporaso *et al.*, 2010) and displayed with R software (Version 2.15.3).

To evaluate the differences in species complexity among samples, beta diversity and unweighted UniFrac diversity were analysed by QIIME software (Version 1.7.0) (Caporaso *et al.*, 2010). The significant differences in bacterial community structure among samples were analysed using MetaStat (White *et al.*, 2009) and LEfSe (Segata *et al.*, 2011). The functional diversity of bacteria in each group was predicted by Tax4Fun (Abhauer *et al.*, 2015).

Results

SEQUENCE ANALYSIS

The bacterial reads of the samples inoculated with *B. xylophilus* after 6 and 12 h (indicated as Bx6h and Bx12h, respectively), and their control groups inoculated with sterile water (C6h and C12h, respectively), as well as healthy wound tissues (Pm) and *B. xylophilus* (Bx), were cut and filtered. After quality control, a total of 1 129 409 bp clean reads, which clustered in 1157 OTUs, were obtained. Among these OTUs, only two (0.17%) OTUs could not be annotated, whereas 1081 (93.43%), 1044 (90.23%), (83.32%), 964 (74.33%), 567 (49.01%) and 113 (9.77%) OTUs were annotated at the phylum, class, order, family, genus and species levels, respectively. The rarefaction curve (Fig. 1) for each group nearly reached saturation, indicating that the sequence data were sufficient and representative.

BACTERIAL DIVERSITY ANALYSIS

To assess the possible alterations in bacterial diversity caused by PWN inoculation, alpha diversity was analysed based on observed species, Chao 1 (richness), Shannon index and Simpson index (diversity) (Table 1). All indices indicated that the bacterial richness and diversity in Group Bx6h were comparable with those of its control group (C6h), while Group Bx12h had significantly lower richness and diversity indices compared to the control group (C12h) ($P < 0.05$, Wilcoxon). Even in the two control groups, the bacterial richness and diversity changed, with the longer time group having greatly increased indices. Based on the sequencing data, we found that the main genera that increased in abundance (Group C12h compared to Group C6h) was *Rhizobacter* and other unidentified genera. Moreover, compared to all the other groups, Group Bx had the lowest bacterial richness and diversity ($P < 0.05$, Wilcoxon).

Fig. 1. Rarefaction curves of observed species from the 6 h pine wood nematode (PWN; *Bursaphelenchus xylophilus*)-inoculation group (Bx6h), the 12 h PWN-inoculation group (Bx12h), their corresponding control groups (C6h, C12h), healthy cortex of *Pinus massoniana* (Pm) and *B. xylophilus* (Bx) group.

Table 1. Alpha diversity indices of bacteria associated with the 6 h pinewood nematode (PWN; *Bursaphelenchus xylophilus*)-inoculation group (Bx6h), the 12 h PWN-inoculation group (Bx12h), their corresponding control groups (C6h, C12h), healthy cortex of *Pinus massoniana* (Pm) and *B. xylophilus* (Bx) group.

Group	Observed species	Shannon	Simpson	Chao1	Coverage
Bx6h	453	6.596	0.979	497.865	0.998
C6h	450	6.505	0.974	486.561	0.998
Bx12h	438*	6.100*	0.960*	488.648*	0.998
C12h	520	7.103	0.986	576.471	0.998
Pm	445	6.338	0.969	465.311	0.998
Bx	101**	2.961**	0.781**	121.701**	0.999

* Statistically different ($P < 0.05$, Wilcox) compared to its control group.

** Statistically different ($P < 0.05$, Wilcox) compared to all the other groups.

BACTERIAL COMMUNITY COMPOSITION

To gain insights into the differences in bacterial communities, we further analysed the individual bacterial taxa in each group and presented the results according to the clusters of Unweighted Pair-group Method with Arithmetic Means (UPGMA) Clustering. As shown in Figure 2,

the PWN-inoculated groups (Bx6h, Bx12h) shared a similar relative abundance of bacterial phyla and clustered together, while the bacterial phyla of Group Bx that we used for inoculation were distinctive from the other five groups.

In Group Bx, a total of 160 OTUs were detected, with Proteobacteria (mean $\pm$ standard error; $98.3 \pm 0.45\%$) being the most abundant phylum, followed by Bacteroidetes ($1.3 \pm 0.31\%$). At the class level, γ -proteobacteria dominated most of the total reads, with a percentage of $96.0 \pm 0.56\%$, and α -proteobacteria ($2.4 \pm 0.30\%$) ranked second (Fig. 3A). At the order level, Xanthomonadales ($36.3 \pm 3.96\%$) ranked first, followed by unidentified γ -proteobacteria ($35.6 \pm 6.00\%$) and Pseudomonadales ($19.2 \pm 1.35\%$) (Fig. 3B). There were five representative families that occupied $> 1\%$ of the total reads: Xanthomonadaceae ($36.2 \pm 3.97\%$), Burkholderiaceae ($35.6 \pm 6.02\%$), Pseudomonadaceae ($19.2 \pm 1.36\%$), Enterobacteriaceae ($4.8 \pm 0.42\%$) and Sphingomonadaceae ($1.6 \pm 0.19\%$) (Fig. 3C). The most abundant genus was *Stenotrophomonas* ($36.2 \pm 3.97\%$), followed by unidentified Burkholderiaceae ($31.9 \pm 5.98\%$), *Pseudomonas* ($19.2 \pm 1.36\%$), *Serratia* ($3.7 \pm 0.23\%$), *Delftia* ($1.6 \pm 0.40\%$), *Novosphingobium* ($1.4 \pm 0.22\%$) and *Chryseobacterium* ($1.0 \pm 0.30\%$) (Fig. 3D). The relative abundance of other genera was less than 1%.

Fig. 2. Relative abundance at the phylum level using the unweighted pair-group method with arithmetic means (UPGMA) clustering. Bx6h, the 6 h pine wood nematode (PWN; *Bursaphelenchus xylophilus*)-inoculation group; Bx12h, the 12 h PWN-inoculation group; C6h, control group of Bx6h; C12h, control group of Bx12h; Pm, healthy cortex of *Pinus massoniana*; Bx, *B. xylophilus*.

Fig. 3. Relative abundance of the top ten bacterial classes (A), orders (B), families (C) and genera (D) for each group. Bx6h, the 6 h pine wood nematode (PWN; *Bursaphelenchus xylophilus*)-inoculation group; Bx12h, the 12 h PWN-inoculation group; C6h, control group of Bx6h; C12h, control group of Bx12h; Pm, healthy cortex of *Pinus massoniana*; Bx, *B. xylophilus*.

For all the other groups from the wound tissues of *P. massoniana* (Bx6h, Bx12h, C6h, C12h and Pm), the average number of OTUs was 684, and the associated bacterial communities were mainly composed of Proteobacteria and Actinobacteria at the phylum level,

α -proteobacteria and unidentified Actinobacteria at the class level (Fig. 2A), and Rhizobial, Pseudonocardiales and Sphingomonadales at the order level (Fig. 2B). The most represented family was Beijerinckiaceae (Fig. 2C). However, the relative abundance at the genus level varied

among different groups (Fig. 2D), especially between the PWN-inoculated groups and their corresponding control groups; thus, we focused on their comparative analysis at the genus level in subsequent analyses.

COMPARATIVE ANALYSIS OF PWN-INOCULATION GROUPS TO CONTROL GROUPS

In Group Bx6h, the most represented genus was *Methylobacterium* ($15.5 \pm 4.29\%$), followed by *Pseudomonas* ($8.0 \pm 3.94\%$). With the time of inoculation prolonged (Group Bx12h), the structure and relative abundance of bacteria at the genus level showed no significant difference, except for the remarkable decrease in *Nocardioides*, which was detected in Group Pm but not in Group Bx.

To identify the change in bacterial communities after inoculation with PWN on the wound surface of *P. massoniana*, we compared the inoculation groups with their corresponding control groups and generated a heatmap together with relative abundance histogram at the genus level (Fig. 4). The comparative results between Groups Bx6h and C6h showed that the most abundant genus from PWN, *Stenotrophomonas*, completely failed to colonise the wound tissues, and the abundance of the other rep-

resentative genus, *Delftia*, also dramatically decreased. Nevertheless, the other top represented genera of PWN (unidentified Burkholderiaceae, *Pseudomonas* and *Serratia*) were significantly enriched ($P < 0.05$, MetaStat), accounting for $2.3 \pm 0.99\%$, $8.0 \pm 3.95\%$ and $0.9 \pm 0.45\%$ of the total reads in this inoculation group, respectively. Comparative analysis between Group Bx12h and the corresponding control group led to similar results except for the remarkably lower relative abundance of *Nocardioides*.

LEfSe (LDA Effect Size), a software for discovering significant differences between groups, was also used, and the results indicated that both PWN-inoculation groups exhibited elevated proportions of *Pseudomonas*, unidentified Burkholderiaceae and *Serratia* (particularly *Serratia liquefaciens*) (Fig. 5). In addition, although with low relative abundance, other genera, such as *Mesorhizobium* (belonging to the family Burkholderiaceae), *Rhizobacter* (controversial taxonomy; our sequencing result classified it as Burkholderiaceae) and *Friedmanniella*, were also enriched in Group Bx6h, while *Yersinia* sp. Ha77 and *Ewingella americana*, both of which belong to Enterobacteriaceae, were enriched in Group Bx12h.

Fig. 4. Heatmap and relative abundance of genera (more than 0.5% of the total reads) with significant differences ($P < 0.05$; MetaStat). The red and black font indicate the significant increase and decrease in abundance of the genera in Group Bx6h and Bx12h, respectively, compared to their control groups, and the up and down arrows in the heatmap demonstrate the specific trend in each pine wood nematode (PWN; *Bursaphelenchus xylophilus*)-inoculation group. The histogram shows the average relative abundance of these genera in each group ($n = 3$). Error bars represent SE of the mean. Bx6h, the 6 h PWN-inoculation group; Bx12h, the 12 h PWN-inoculation group; C6h, control group of Bx6h; C12h, control group of Bx12h; Pm, healthy cortex of *Pinus massoniana*; Bx, *B. xylophilus*.

Fig. 5. Relative abundance of bacterial genera in pine wood nematode (PWN; *Bursaphelenchus xylophilus*)-inoculation groups (Bx6h and Bx12h, red) and control groups (C6h and C12h, green) based on LEfSe results. A: Group Bx6h vs Group C6h; B: Group Bx12h vs Group C12h. Bars represent linear discriminant analysis (LDA) scores. The threshold value was set to 3. The lowercase letters f, g and s in front of the bacterial names represent family, genus and species level, respectively. The genera and species of the same family were framed in red box with the family name nearby.

FUNCTIONAL PREDICTION

We used Tax4Fun based on the 16S Silva database to predict the functional diversity of the bacteria in each group. A total of 6471 functional orthologues were predicted, assigning to 43 level 2 KEGG pathways, with membrane transport, carbohydrate metabolism, amino acid metabolism and translation having greater abundance of related genes. A heatmap was made from the top 25 KEGG orthologue (KO) groups according to functional annotation and abundance, and all groups were clustered based on functional relative abundance. As expected, the functions of the bacteria associated with Group Bx were significantly different from those associated with the other groups (data not shown), with higher KO abundance in metabolism-related pathways such as ‘enzyme families’ and ‘glycan biosynthesis and metabolism’, environmental information processing pathways such as ‘membrane transport’ and ‘signal transduction’, and cellular processes pathways such as ‘cell motility’ and ‘cellular community prokaryotes’. Among the other groups from the wound tissues of *P. massoniana*, the bacterial functions of Group Bx6h were more similar to those of its control group and the other two non-PWN-inoculation groups, while the bacterial functions of Group Bx12h clustered farther from all the other groups (Fig. 6). As seen from the heatmap, Group Bx12h had higher KO abundance in ‘carbohydrate metabolism’, ‘xenobiotic biodegrada-

tion and metabolism’, ‘amino acid metabolism’, ‘lipid metabolism’, ‘metabolism of terpenoids and polyketides’, ‘nucleotide metabolism’, ‘enzyme families’, ‘transport and catabolism’ and ‘replication and repair’ pathways. In level 2 KEGG pathways, both Group Bx6h and Group Bx12h had no significant alteration in KO abundance compared to their control groups. However, Group Bx12h had significantly higher KO abundance in the ‘metabolism of cofactors and vitamins’ and ‘nucleotide metabolism’ pathways than Group Bx6h.

Discussion

Generally, the bacterial populations and diversity inside trees increase with disease progression (Xie & Zhao, 2008), but little is known about the bacteria in wound tissues in the initial stage before the pathological response of pines to PWN invasion can be seen. Our study illustrates the changes in bacterial communities in the wound tissues of *P. massoniana* after inoculation with PWN. The results showed that the bacterial richness and diversity changed little after 6 h but decreased significantly after 12 h compared to their control groups. However, obvious alterations in some of the predominant bacteria carried by PWN occurred within 6 h; in particular the unidentified Burkholderiaceae, *Pseudomonas* and *Serratia* ge-

Fig. 6. Heatmap of the top 25 KEGG orthologous (KO) groups according to the functional annotation and abundance of level 2 KEGG pathways based on Tax4Fun. Bx6h, the 6 h pine wood nematode (PWN; *Bursaphelenchus xylophilus*)-inoculation group; Bx12h, the 12 h PWN-inoculation group; C6h, control group of Bx6h; C12h, control group of Bx12h; Pm, healthy cortex of *Pinus massoniana*; Bx, *B. xylophilus*.

nera were enriched but the abundance of *Delftia* and *Stenotrophomonas* were either reduced or excluded.

Unlike the increasing population dynamics of bacteria in disease stages, the bacterial colonies in the adjacent xylem of inoculation sites hardly changed after 3 h and were generally lower than those in the samples with disease symptoms (Roriz *et al.*, 2011), probably because there is a barrier outside the xylem, which is the defence response of the host, leading to little change in bacterial communities in the xylem. In our study, the inoculation of PWN apparently directly changed the bacterial communities in the wound tissues of PWN, particularly after 12 h, which may be enough time for adaptive bacteria to survive and proliferate and for host pine to eliminate unsuitable bacteria. Interestingly, Group C12h had the highest bacterial richness and diversity, indicating that even without PWN inoculation, the bacterial communities in the wound tissues might change. Although the majority of newly added bacteria remained unidentified in this group, the increasing richness was partly due to *Rhizobacter*, which was also observed in healthy Group Pm but with lower abundance. In addition, *Rhizobacter* was more abundant

in both PWN-inoculation groups than Group Pm; thus, we suspect it might be beneficial for pine trees to confront injuries or resist the invasion of PWN. In support of this suggestion, Dhanasekar & Dhandapani (2012) proposed that *Rhizobacter* is a nitrogen-fixing bacteria and is mainly involved in the biological control of pathogens, nutrient cycling and seedling establishment.

In Group Bx, the dominant bacterial genera were *Stenotrophomonas*, unidentified Burkholderiaceae, *Pseudomonas*, *Serratia*, *Delftia*, *Novosphingobium* and *Chryseobacterium*. Among these bacteria, *Stenotrophomonas* was the most dominant genus, in accordance with previous studies (Tian *et al.*, 2010; Wu *et al.*, 2013), and *Stenotrophomonas* was also detected in PWN obtained from *P. massoniana*. *Pseudomonas*, *Serratia* and Burkholderiaceae have been widely reported in association with PWN and are able to induce PWD symptoms (Oku *et al.*, 1980; Han *et al.*, 2003; Guo *et al.*, 2006, 2007; Proença *et al.*, 2010; Vicente *et al.*, 2011, 2012). Meanwhile, *Serratia*, namely, *S. marcescens*, was associated with *M. alternatus*, and secondary metabolites of *S. marcescens* are able to degrade lignin (Fu, 2017). In our study,

we also detected *S. marcescens* in PWN, but with a low proportion ($0.1 \pm 0.02\%$). Cheng *et al.* (2013) and Wang *et al.* (2019) found that *Delftia* and *Novosphingobium* were dominant in PWN isolated in Zhejiang Province in China, and Liu *et al.* (2017) identified *Delftia*, *Pseudomonas*, *Stenotrophomonas* and *Rhizobium* as the dominant species on the surface of PWN in the USA. *Chryseobacterium* was also reported to be one of the main species carried by the PWN from Japan (Ju *et al.*, 2008) and the USA (Proença *et al.*, 2014). It is plausible that bacteria carried by PWN varied in different studies because of multiple factors, such as geographic area, *Pinus* species, feeding fungus, isolation methodologies and surface sterilisation. To avoid eliminating the potential PWD-related bacteria, the PWN we used here were obtained without sterilisation because a considerable number of bacterial species are present on the cuticle surface of PWN (Roriz *et al.*, 2011; Vicente *et al.*, 2011), and superficially associated bacteria can also potentially be involved in PWD (Li, 2008).

In Group Pm, the bacterial community was dominated by Proteobacteria and Actinobacteria at the phylum level. The top three genera were *Methylobacterium*, *Sphingomonas* and unidentified *Beijerinckiaceae*, all of which belong to Proteobacteria. A recent study also reported that the most dominant endophytic bacteria in healthy, as well as in diseased, *P. massoniana* is Proteobacteria (Li *et al.*, 2018). However, the corresponding genera were different, and the second most dominant phylum was Bacteroides, which ranked third in our study. These differences could be explained by differences in sampling location, sampling area and tree age. In addition, several studies have confirmed the existence of endophytic bacteria in pine trees, with *Methylobacterium* being the major genus (Pirttilä *et al.*, 2000, 2008), possibly simply because it is a beneficial endophytic bacteria for the growth of pine seedlings (Pohjanen *et al.*, 2014).

After inoculation with PWN, unidentified Burkholderiaceae, *Pseudomonas* and *Serratia* (especially *S. liquefaciens*) were introduced to the wound surface together with PWN and significantly enriched within at least 6 h, while interestingly the most dominant genus, *Stenotrophomonas*, could not colonise wound tissues and the abundance of one of the representative genera, *Delftia*, was reduced, indicating that unidentified Burkholderiaceae, *Pseudomonas* and *Serratia* adapted to the environment containing secondary metabolites from the host but *Stenotrophomonas* and *Delftia* did not. For the above result, we may doubt that the increases in Burkholderiaceae, *Pseudomonas* and *Serratia* resulted from the in-

roduction of PWN, but the abundance of these bacteria also increased in Group Bx12h compared to Group Bx6h, confirming that they were indeed enriched in the wound tissues and may play a role in the initial infection activities. In summary, these bacteria could be considered as biomarkers to understand the development of PWD in this specific stage.

As terpenoids (especially α -pinene) are defence compounds of pine trees and are detrimental to the reproduction of the PWN (Kong *et al.*, 2007), α -pinene degradation is important for the survival and invasion of PWN. It was reported that *Pseudomonas* is one of the main strains capable of degrading α -pinene; it not only survives the stress of α -pinene but also utilises α -pinene and other secondary metabolites of pine, including benzoate, as a carbon source for growth, whilst *Stenotrophomonas* cannot survive under the stress of benzoate and grows slowly in LB medium containing α -pinene (Cheng *et al.*, 2013). A recent study observed similar results; the abundance of *Pseudomonas* sp. carried by PWN increased, while the abundance of *Stenotrophomonas* and *Delftia* spp. decreased when PWN was fumigated with α -pinene (Wang *et al.*, 2019), which may explain the enrichment of *Pseudomonas* and the reduction or even exclusion of *Delftia* and *Stenotrophomonas* in our study. However, we could not ignore the fact that one species of *Stenotrophomonas*, namely, *S. maltophilia*, was related to the strong virulence of PWN (Wu *et al.*, 2013), and when *S. maltophilia* NSPmBx03 was treated with PWN, many cell wall degradation-related and detoxification-related genes, including pectate lyase, glutathione S-transferase, ATP-binding cassette transporter and cytochrome P450, might be upregulated, and the virulence of PWN might be enhanced (He *et al.*, 2016). In addition, some *Stenotrophomonas* spp. were reported as being able to degrade aromatic compounds and other xenobiotics (Mangwani *et al.*, 2014; Tiwari *et al.*, 2016), which seems beneficial for PWN to invade its host. However, other strains of *S. maltophilia* might inhibit PWN hatching (Tian *et al.*, 2015) or possess nematicidal activity against PWN (Huang *et al.*, 2009), leading to the opposite result. A similar controversy was also presented by *Serratia*: many species exhibited high toxicity against PWN *in vitro* (Paiva *et al.*, 2013) but some were able to produce copious cellulose, form biofilms, resist reactive oxygen species, tolerate growth in the presence of xenobiotic/organic compounds and not only colonise themselves (Nascimento *et al.*, 2018; Vicente *et al.*, 2012, 2013a, 2016b) but also assist PWN survival under prolonged oxidative stress con-

ditions (Vicente *et al.*, 2013b, 2016b). Considering these contradictory results on different strains within the same genera, the role of these bacteria in PWN and PWD needs more investigation. Interestingly, within the *Serratia* genus, *S. liquefaciens* was reported for the first time in our study to be related to PWN. It was relatively abundant (3.3%) in Group Bx and enriched in the wound tissues after 6 h of PWN inoculation. Although its detailed role in PWN remains to be further studied, this species has been described to produce volatile organic compounds and thus could promote plant growth and attract *Caenorhabditis elegans* (Grewal & Wright, 1992; Wang, 2018).

To predict the functions of bacteria, we used the Tax4Fun approach. Because of the limitation of this method, we could not identify the exact role of each bacterium, but the results of this section could suggest that the alterations in bacterial composition potentially lead to shifts in their functional pathways. This result was more obvious in the longer inoculation time group (Group Bx12h) in our study and was reflected mainly in the increase in KO abundance in multiple metabolism-related pathways such as ‘carbohydrate metabolism’, ‘xenobiotics biodegradation and metabolism’, ‘amino acid metabolism’, ‘lipid metabolism’, and ‘metabolism of terpenoids and polyketides’, which potentially indicates that related bacteria may play a role in the invasion of PWN at 12 h. Further studies are needed to determine the specific role of these bacteria in pine trees *in vivo*.

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